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N⁸-acetylspermidine as a potential plasma biomarker for Snyder-Robinson syndrome identified by clinical metabolomics

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Abstract Clinical metabolomics has emerged as a powerful tool to study human metabolism in health and disease. Comparative statistical analysis of untargeted metabolic profiles can reveal perturbations of metabolite levels in diseases and thus has the potential to identify novel biomarkers. Here we have applied a simultaneous genetic-metabolomic approach in twin boys with epileptic encephalopathy of unclear etiology. Clinical exome sequencing identified a novel missense mutation in the spermine synthase gene (*SMS*) that causes Snyder-Robinson syndrome (SRS). Untargeted plasma metabolome analysis revealed significantly elevated levels of N⁸-acetylspermidine, a precursor derivative of spermine biosynthesis, as a potential novel plasma biomarker for SRS. This result was verified in a third patient with genetically confirmed SRS. This study illustrates the potential of

metabolomics as a translational technique to support exome data on a functional and clinical level.

Introduction

Mass spectrometry (MS) based screening for biomarkers of inborn errors of metabolism (IEM) is now routine and applied in newborn screening programs as well as in selective work-up of IEM (Chace 2001). MS-based metabolomics represents a comprehensive, quantitative and simultaneous analysis of all small molecule metabolites in a defined biofluid and thus reflects its distinct metabolic composition (Patti et al 2012; Moco et al 2013). Recent advances in instrumental and computational technologies have enabled clinical metabolomics to

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Lucia Abela and Luke Simmons contributed equally to this work.

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emerge as a powerful tool for the identification of novel biomarkers and distinct metabolic profiles in IEM (Wikoff et al 2007; Shlomi et al 2009; Moco et al 2013).

Snyder-Robinson syndrome (SRS) is a rare X-linked recessive disorder. By linkage analysis the disease-causing gene was mapped to the chromosomal region Xp.21.3-p22.12, harboring the spermine synthase gene (*SMS*) with first mutations identified in 2003 (Cason et al 2003). Spermine is a polyamine that is involved in several key cellular processes including proliferation, protein and nucleic acid synthesis, cell adhesion and signaling and ion channel regulation (Pegg and Michael 2010). Spermine synthase (*SMS*; EC: 2.5.1.22) converts spermidine to spermine and belongs to a group of aminopropyltransferases (Pegg and Michael 2010; Sowell et al 2011) (Fig. 1).

SRS is characterized by moderate to severe intellectual disability, unsteady gait, and hypotonia (Snyder and Robinson 1969). To date, only four families with SRS have been described, indicating that the syndrome is either very rare or underdiagnosed. Reevaluation of the original and novel families led to the delineation of a syndromic phenotype additionally characterized by facial dysmorphism, asthenic body build, bone abnormalities including osteoporosis and kyphoscoliosis, nasal dysarthric, coarse, or absent speech, high or cleft palate, urogenital abnormalities, mild short stature and seizures (Arena et al 1996; Cason et al 2003; de Alencastro et al 2008; Becerra-Solano et al 2009; Peron et al 2013).

Here, we report the identification of N⁸-acetylspermidine as a potential novel plasma biomarker for spermine synthase

deficiency through untargeted plasma metabolomics analysis of twin boys and a further unrelated previously published patient (Peron et al 2013).

Clinical report

The male monozygotic twins were delivered by cesarean section at 37 5/7 gestational week after a 1st uncomplicated pregnancy of healthy, non-consanguineous parents. Apgar scores were 8-8-9 in both. Birth weight, length and head circumference were below the 3rd percentile in both twins. Cytomegalovirus (CMV) PCR from the newborn blood spot screening was negative in twin A, while the card could not be analyzed in twin B for technical reasons. In twin B, CMV PCR in urine was positive. However, no other evidence for congenital CMV infection was found, including normal ophthalmological findings. Brain stem audiometry at the age of 4 months was normal in twin A and revealed a mild conduction defect in twin B. Behavioral observation audiometry was normal in twin B at age 2 years. Early development was delayed and both twins presented with axial hypotonia and muscular hypertonia of the lower extremities. Dysmorphic features became more apparent during the second year of life. At the age of 12 months, recurrent irregular myoclonic jerks were observed in both twins and serial infantile spasms in twin A. Twin B showed photosensitivity with myoclonic jerks, twin A had hypsarrhythmia in sleep and serial spasms at awakening. Myoclonic seizures were drug-refractory and both

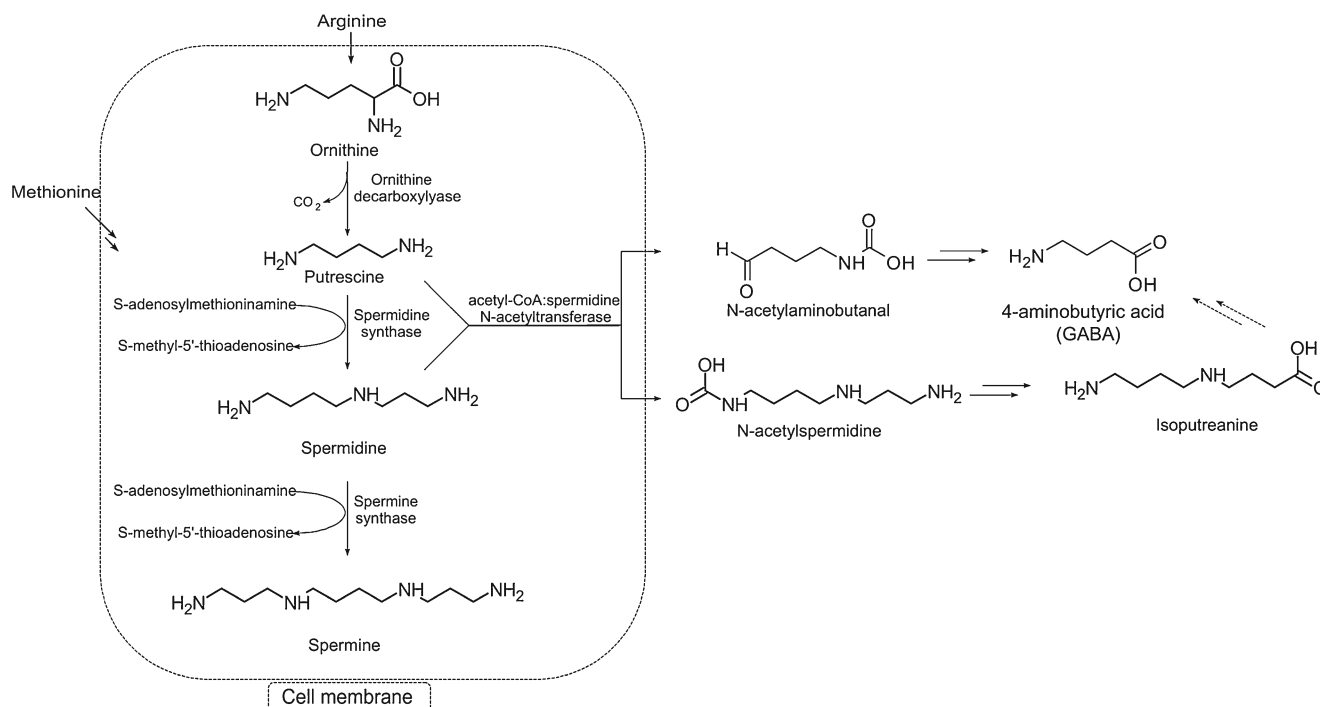


Fig. 1 Polyamine biosynthesis and conversion pathway, highlighting selected metabolites discussed in the text

boys suffered additional atonic and tonic seizures as well as atypical absences in the further course. At the age of 15 months, along with recurrent respiratory infections, they developed a severe encephalopathy with frequent seizures and developmental regression with reduced social and visual interaction. From the age of 17 months both twins developed a distinct choreo-athetotic movement disorder that gradually improved over the following 6 months. At the age of 23 months, twin B suffered from non-traumatic left-sided clavicular fracture, both showed osteopenia. At the last clinical exam at the age of 29 months, development was severely affected with absence of speech, active sitting, and grasping. Both boys still suffered from generalized myoclonic jerks and asymmetric tonic seizures and displayed abnormal EEGs.

Head circumference was -2.9 SD in both with plateauing of head growth since the age of 18 months. Magnetic resonance imaging of the brain at age 13 (twin A) and 14 months (twin B) respectively, showed a thin corpus callosum and slight cerebral atrophy in twin A, while twin B had isolated bilateral pallidal hyperintensity. Screening tests for metabolic diseases were all normal (details in [Supplementary material](#)). Details on the clinical course are given in the [Supplementary material](#).

Methods and materials

Parents of all three patients had given full written informed consent for participation in a study on metabolic-genetic research into early onset epileptic encephalopathies (EE) and a metabolome study, respectively. Both studies have been approved by the Institutional Review Board of the Kanton of Zurich. The plasma sample of the third SRS patient was submitted upon specific request. Control samples included seven patients (age range 1.5–13.5; mean 7.7 ± 5.87 , median 9.2 years) from the above mentioned EE study cohort in which exome sequencing revealed disease-causing mutations other than *SMS* or were inconclusive. We further included six age- and sex-matched and six sex-matched healthy controls of similar age (range 32–45 months; mean 40 ± 4.8 ; median 41 months).

Reagents

All reference compounds, ultra LC-MS grade solvents and reagents for the metabolomics analysis were purchased from Sigma-Aldrich AG (Buchs, Switzerland).

Genetic studies

Genomic DNA was extracted from EDTA blood. Zygosity testing was performed using the PowerPlex™ 16

microsatellite kit (Promega Inc.) in both twins. High resolution copy number profiling was performed with the CytoscanHD microarray (Affymetrix Inc.). Targeted exome sequencing on 4813 known disease genes was performed using the TruSight™ One Sequencing Panel (4813 Genes, Illumina Inc.) with paired-end sequencing (MiSeqReagent Kit v3, 200 Fwd-200 Rev) on a MiSeq System (Illumina Inc.). Filtering of variants is described in [Supplementary material](#). The candidate *SMS* variant was confirmed and tested for segregation by Sanger sequencing. Maternal X-chromosome inactivation pattern at the androgen receptor was investigated as described (Lau et al 1997).

Metabolomics data acquisition and preprocessing

Samples were analyzed by liquid chromatography-mass spectrometry (LC-MS; Dionex Ultimate XRS3000 UHPLC coupled to a Q-Exactive high resolution, accurate mass spectrometer (Thermo Scientific, Sunnyvale, CA, USA)). Chromatographic separations were achieved using a 2.1×100 mm Kinetex HILIC column (Phenomenex, Torrance, CA, USA). Mobile phases used were: A) 50 % acetonitrile in 5 mM ammonium formate (pH 3.0), and B) 90 % acetonitrile in 5 mM ammonium formate (pH 5.0). Total run time was 15 min with a 10 min linear gradient running from 100 % B to 100 % A. Solvents were pumped at 400 μ L/min, with a column temperature of 30 °C and sample chamber held at 5 °C. Mass spectrometric measurements were acquired in positive and negative ionization modes through the heated electrospray ionization (HESI) source.

The untargeted metabolomics data set was acquired with the MS detector in full-scan mode (Full-MS) with data-dependent (dd-MS2) acquisition of fragment ions from the top-5 most abundant ions per scan. Detector settings can be found in the [Supplementary material](#).

Data were collected in Xcalibur (Thermo) and preprocessing was performed in R (x64, v3.1.0) the free software environment for statistical computing and graphics (<http://www.r-project.org/>). Feature detection and retention time correction were done using XCMS (Smith et al 2006; Tautenhahn et al 2008). Peak list annotation was done in CAMERA (Kuhl et al 2012). All data features of interest were manually inspected for peak shape and alignment, and then mined against the Metlin database (Smith et al 2005) and the Human Metabolome Database (<http://www.hmdb.ca/>). Date of the last database access was March 27, 2015 for both Metlin and HMDB.

Statistical analysis

Data pretreatment included 1) noise filtering and missing value imputation, where negative data values or features missing from at least 80 % of data were eliminated 2) data

normalization, achieved by calculating the sum of all variables within a spectrum and by factoring each spectral sum to a value of 1. Each variable is then transformed as a fraction of the total spectral sum. All data preprocessing, data scaling (e.g., *pareto*) and univariate analysis were performed in R utilizing the MUMA package (Gaude et al 2013). Multivariate data analysis was performed by SIMCA v13.0.3 (Unimetrics, Malmö, Sweden). Data matrices containing mass over charge (m/z), and normalized ion signal intensity were analyzed with the unsupervised principle components analysis (PCA; Figure S1). Orthogonal projection to a latent structure-discriminant analysis (OPLS-DA) was used to evaluate and rank the differential metabolite expression profiles (Table S1 and [Supplementary material](#)). Relative metabolite ratios were calculated by comparing the integrated area under curve for the target metabolite. Receiver operating characteristic (ROC) analysis and false-discovery rates were calculated in GraphPad Prism 6 (Fig. S2; GraphPad Software Inc.)

Determination of polyamine ratios

Samples

Lymphoblast cell cultures were established from patient EDTA-blood according to standardized protocols. Patient lymphoblasts were grown in Gibco RPMI-1640 Media-20 % FBS, 1 % antibiotic/antimycotic in a 10 % CO₂ regulated incubator with humidity. Cultures were pelleted by centrifugation at 150×g for 3 min, washed in ice cold phosphate buffered saline and centrifuged at 150×g for 3 min. The cell pellets were suspended in 500 µL of 0.1 % formic acid in methanol, sonicated for 5 min and centrifuged at 20,000×g for 15 min at 4 °C. The supernatant was aspirated into a clean tube and dried under reduced atmosphere. The resultant organic fraction was resolved in 10 % acetonitrile in ultra-grade water with 0.1 % formic acid (FA) and 0.02 % heptafluorobutyric acid (HFBA).

LC-MS analysis of polyamine ratios

Analysis of the spermine/spermidine ratio was done according to published methods with minor modifications (Sowell et al 2011). Chromatography was performed on a Thermo Scientific Dionex Ultimate XRS 3000 UHPLC system using a C8 column (Supelco Ascentic Express C8 2.1×100 mm) at 30 °C. A 5 µL injection volume was used for analysis with a solvent system consisting of mobile phase A (0.1 % FA and 0.02 % HFBA in water), and mobile phase B (0.1 % FA and 0.02 % HFBA in acetonitrile). Spermine and spermidine were separated using a linear gradient of 10 to 40 % mobile phase B over 10 min with a flow rate of 0.3 mL/min. Mass spectrometric data were acquired on a Thermo Scientific Q-Exactive Orbitrap operating in positive ionization mode.

Results

Genetic analysis

Targeted exome sequencing in twin A covered about 95 % of the targeted region with ≥20 independent sequence reads and revealed a total of 105 private non-synonymous coding and splice site variants in known disease genes. After filtering of the variants found, four candidate variants were further investigated by Sanger sequencing (SOS1 (p.Leu356Ile); SPTAN1 (c.238-9C>T); SMS (p.Arg130Cys); EFHC1 (p.Phe229Leu)). While the autosomal variants in SOS1, SPTAN1, and EFHC1 were found inherited from the healthy father and thus considered likely benign, the hemizygous missense mutation within exon 5 of the X-linked spermine synthase gene (*SMS*: c.388C>T (p.Arg130Cys); hg19 chrX:21995237;) was compatible with X-linked recessive inheritance and was shown to be inherited from the healthy mother. The mutation was absent in both maternal grandparents and thus likely occurred *de novo* in the carrier mother. Maternal X-inactivation in blood was found to be mildly skewed, with one allele active in 83 % of the cells and the other allele in 17 %. Since the mother is a healthy carrier, we assume that the wild-type allele is preferentially active. The mutation was not reported previously and affects an evolutionary conserved amino acid. Moreover, five of six applied in silico programs predicted a deleterious effect of the SMS mutation (SIFT: score 0 (damaging); PhyloPhen: score 0.9990/0.7180 (D probably damaging/P possibly damaging); LRT: score 0 (deleterious); Mutation taster: score 1 (disease causing); Mutation assessor: score 2.325 (medium → predicted functional); FATHMM: score -0.6300 (tolerated, values lower than -1.5 are predicted as damaging). Information on the SMS mutation modeling is included in the [Supplementary material](#) and in Fig. S3.

Metabolomics analysis

The details of the metabolomics data preprocessing can be found in the [Supplementary material](#). PCA of the resultant data matrix revealed differential clustering of the SRS cohort against the control cohort with 97 % of the variation within the training set explained by the model and 90 % of the variation in the training set predicted by the model according to cross validation in the 26th component (R2X (cum)=0.97; Q2 (cum)=0.90). Application of supervised OPLS-DA allowed a detailed differential expression analysis between the SRS and control cohorts (R2X (cum)=0.71; R2Y (cum)=0.99; Q2 (cum)=0.99). An overall false-discovery rate (FDR) of 0.01 was calculated by an empirical Bayesian method based on moderated t-statistics (Schwender and Ickstadt 2008).

Spermine to spermidine ratio and spermine synthase deficiency

Calculation of the ratio of spermine to spermidine ratio in cultured lymphoblasts derived from twin A and B gave values of 0.02 and 0.05, while the ratio was 0.12 and 0.10 in two controls respectively. These values are indicative of a deficiency in spermine synthase and correspond to those previously established for SRS (Sowell et al 2011). The Western blot analysis of cell lysates in the twin brothers further demonstrated the absence of spermine synthase thus confirming the diagnosis on a protein level (Fig. S4).

N⁸-acetylspermidine as a novel potential biomarker for Snyder-Robinson syndrome

The results of the discriminant analysis (OPLS-DA; Table S1) identified several compounds directly corresponding to polyamine metabolism in the SRS patient plasma samples: N⁸-acetylspermidine, spermidine, isoputrescine and ornithine (Figs. 2 and 3). Identification of N⁸-acetylspermidine was confirmed by comparison of accurate-mass signal ($C_9H_{21}N_3O$ cal'd [M+H]⁺ 188.17574; Obs. 188.17526 Δ ppm 2.4), retention time and MS/MS fragmentation pattern with that of commercially available reference material (Figs. S5 and S6). Integration of the area under curve for this ion peak revealed a greater than threefold relative increase in the SRS samples versus the control samples (Fig. 3). Statistical significance was confirmed by receiver operating curve (ROC) analysis (Fig. S2).

Discussion

SRS is a rare X-linked disorder that was hitherto diagnosed by molecular analysis of the *SMS* gene and/or analysis of the spermine/spermidine ratio in patient lymphoblasts based on an LC-MS/MS assay (Sowell et al 2011). By means of a non-targeted metabolomics approach, we were able to identify a novel potential plasma biomarker for SRS which requires a single blood plasma sample. Rigorous statistical analysis of

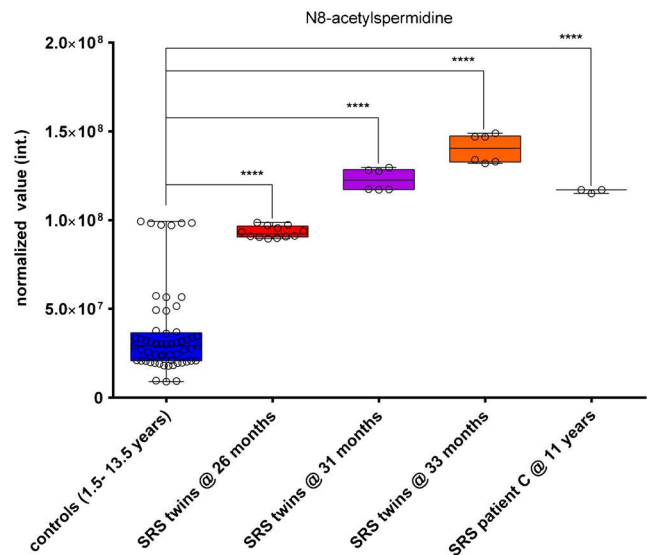


Fig. 3 Box plots showing normalized N⁸-acetylspermidine ion intensities for controls ($n=19$) and SRS samples with longitudinal sample collection for SRS twins A and B ($n=9$). Data for each sample was acquired in technical triplicates. **** indicates p -value < 0.001

the global metabolomics data revealed several significantly altered metabolites involved in spermine/spermidine metabolism (Table S1). Among them, the peak intensity of N⁸-acetylspermidine was significantly higher in the twin patients and a third unrelated SRS patient (Peron et al 2013) compared to 19 controls. Spermine synthase deficiency leads to accumulation of spermidine that is converted to N⁸-acetylspermidine and exported from the cell. Isoputrescine results from conversion of N-acetylspermidine and serves as a precursor for 4-aminobutyric acid (GABA), the most important inhibitory neurotransmitter. The function of spermine and spermidine in SRS disease etiology is not yet fully elucidated, but it might be speculated that the altered spermine/spermidine ratio may severely affect the balance between excitatory and inhibitory mechanisms and result in cerebral dysfunction such as epilepsy. Spermine and spermidine regulate brain glutamate receptors including N-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors that are involved in excitatory synaptic transmission (Pegg 2009; Igarashi and Kashiwagi 2010). Spermine

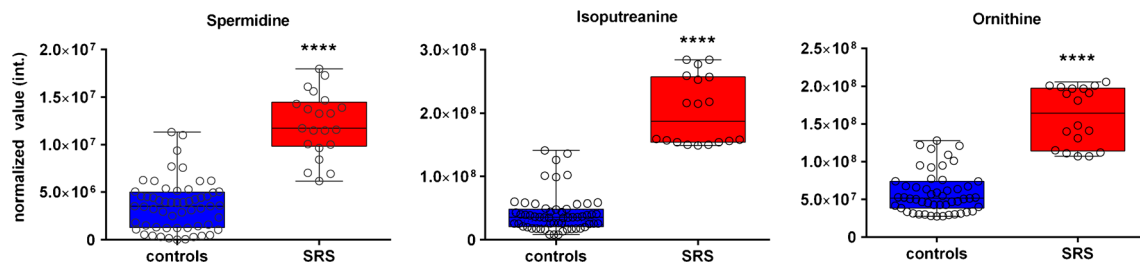


Fig. 2 Box plots for spermidine, isoputrescine, and ornithine showing significant relative variation ($p < 0.001$) between control (blue) and SRS (red) plasma samples. Data for each sample was acquired in technical triplicate with three longitudinal samples (July/December 2014,

February 2015) collected for SRS twins. All three metabolites correspond to the polyamine metabolic pathway given in Fig. 1. **** indicates p -value < 0.001

activates the endogenous phosphorylation and function of the GABA_A receptor (SidAhmed-Mezi et al 2010). It was shown that GABA_A receptor phosphorylation is significantly reduced in epileptogenic human tissue (SidAhmed-Mezi et al 2010). Spermidine itself mediates pro-epileptic effects by increased NO-synthesis in an experimental rat model (Stojanovic et al 2010). Furthermore both spermine and spermidine are potent blockers of voltage-gated Na⁺ channels and their pharmacological depletion leads to increased epileptogenic activity in pyramidal neurons (Fleidervish et al 2008). Modulation of NMDA receptors was further implicated in the etiology of intellectual disability (Igarashi and Kashiwagi 2010; Pegg 2014). Other SRS phenotypic aspects such as osteoporosis and deafness are explained by the involvement of spermine and spermidine in the regulation of osteoclastic activity and endocochlear potential homeostasis (Wang et al 2009; Yamamoto et al 2012).

In our twin patients, the diagnosis of SRS was confirmed by clinical exome sequencing. The mutation Arg130Cys was not described previously and is predicted to diminish enzymatic activity by decreasing dimer stability and affecting the structure of the adjacent spermine binding site. The phenotypic aspects of our patients match those of previously published SRS patients. However, some of the typical features including dysarthric speech and movement disorder may only evolve with age and thus hamper a timely diagnosis (de Alencastro et al 2008; Kesler et al 2009; Peron et al 2013). Peron et al. recently described a patient with SRS and onset of hypsarrhythmia at the age of 1 year (Peron et al 2013). The phenotype of our patients with early onset epileptic encephalopathy further expands the clinical spectrum and confirms that early-onset myoclonic seizures may represent a cardinal feature of those SRS patients presenting with epilepsy as suggested by Peron et al (2013).

In summary, we provide evidence for a potential novel plasma biomarker in SRS and report on the pathogenicity of a novel Arg130Cys mutation in SRS by a combined multi-omics approach. This study illustrates the potential of metabolomics as a translational technique to support exome data on a functional and clinical level.

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Compliance with ethic guidelines All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

Conflict of interests None.

Informed consent Consent was obtained from all patients or their legal guardians for being included in the study.

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